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Tunic Morphology and Cellulosic Components of Pyrosomas, Doliolids, and Salps (Thaliacea, Urochordata)

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Abstract. The morphology and cellulosic composition of the tunic was studied in pelagic tunicates (3 pyrosomas, 2 doliolids, and 13 salps). The tunic is transparent and gelatinous, consisting of an electron-dense cuticular layer with a fibrous tunic matrix. The thickness and density of the cuticular layer and of the tunic matrix differ from species to species. In some salps, the cuticular layer has numerous minute protrusions that are structurally identical to those found in several ascidians. Free mesenchymal cells (tunic cells) are distributed in the tunic. Whereas the number of tunic cells in the pyrosomas is similar to that in ascidians, there are many fewer tunic cells in doliolids and salps. These differences may be caused by the different functions of the tunic in each group. The existence of cellulose in the tunic was confirmed using electron diffraction in all of the species studied thus far. Their diffractograms indicate that the cellulose microfibrils consist of nearly pure 1β of the allomorph. These results show that tunic morphology and cellulosic composition are similar in ascidians and thaliaceans (pyrosomas, doliolids, and salps). The tunic is considered to be a homologous tissue in these animals, and their most recent common ancestor would have possessed this tissue.

Introduction

Members of the phylum Chordata are characterized by having a notochord during some stage of development. Urochordata (also called Tunicata) is one of three subphyla in the phylum Chordata. The name Tunicata is derived from the unique integumentary tissue, called the tunic, that entirely covers the epidermis. The Urochordata includes three classes; all of the species possess tunic in the classes Ascidiacea and Thaliacea, whereas the presence of tunic is not well documented in the class Appendicularia. The tunic is a peculiar tissue among metazoans because of its cellulosic components (De Leo *et al.,* 1977) and the presence of free-living cells (tunic cells) in the tunic, that is, outside the epidermis. To date, the biology and biochemistry of the tunic have been studied mainly in ascidians, sessile forms of tunicates, but they have not been well investigated in pelagic tunicates.

In ascidians, many types of tunic cells have been described, and they are involved in various biological functions, such as phagocytosis (De Leo *et al.,* 1981; Hirose *et al.,* 1994), conduction of impulses (Mackie and Singla, 1987), contractility of the tunic (Hirose and Ishii, 1995), bioluminescence (Aoki *et al.,* 1989; Chiba *et al.,* 1998), photosynthetic symbiosis (Hirose *et al.,* 1996b), and allorecognition (Hirose *et al.,* 1997c). The tunic is overlaid by a cuticular layer that sometimes has a subcuticular layer beneath it. In several ascidian species, the cuticular surface has numerous minute protrusions that are 100 nm high or less. Descriptions of the cuticular fine structures in 116 ascidian species indicate that the presence or absence of cuticular protrusions has phylogenetic significance (*cf.* Hirose *et al.,* 1997b). Little information has been accumulated on the tunic of pelagic tunicates, such as pyrosomas, doliolids, and salps (reviewed in Welsh, 1984, and Bone, 1998). In this study, we investigated the tunic morphology of thaliaceans, with special attention to the distribution of the tunic cells and the fine structure of the cuticule.

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Almost all ascidians studied to date have been found to contain cellulose I microfibrils in the tunic (Yamamoto *et al.*, 1989; Van Daele *et al.*, 1992; Kimura and Itoh, 1996; Okamoto *et al.*, 1996). In pelagic tunicates, however, research has thus far shown cellulose I microfibrils with high crystallinity in the tunic of only one species of Salpidae, *Salpa fusiformis* (Belton *et al.*, 1989). It is not yet known whether other pelagic tunicates can make cellulose. Our study also focuses on the existence and characterization of cellulose in pelagic tunicates.

This report deals with the tunic morphology and cellulosic components of 18 thaliacean tunicates from all orders of Thaliacea. The results provide information valuable for better understanding the diversity and evolution of the tunic in conjunction with the phylogeny of tunicates.

Materials and Methods

Sample collection and fixation

We examined 3 species of pyrosomas, 2 species of doliolids, and 13 species of salps (see Table I), which were collected in several net tows taken southeast of Tokyo, Japan (34°11′–35°04′ N, 139°06′–143°32′ E). Apparently intact animals were sorted from the samples on board ship and prefixed immediately in 2.5% glutaraldehyde-0.45 *M* sucrose-0.1 *M* cacodylate (pH 7.4) at room temperature. Large species, such as *Thetys vagina*, *Salpa fusiformis*, and *Iasis zonaria*, were dissected in the fixation medium, and the tunic near a gill bar was used for the following examinations.

Microscopy for the observation of tunic morphology

After a brief rinse in 0.45 *M* sucrose-0.1 *M* cacodylate (pH 7.4), the specimens were postfixed in 1% osmium tetroxide-0.1 *M* cacodylate for 1.5 h, dehydrated through an ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in low-viscosity epoxy resin. Thick sections were stained with 1% toluidine blue for light microscopy. Thin sections were double stained and examined in a Hitachi HS-9 transmission electron microscope at an accelerating voltage of 75 kV.

In some of the prefixed specimens, the tunic was isolated from the other tissues and observed using a light microscope equipped with Nomarski differential interference contrast (DIC) and phase contrast optics.

Microscopy for the analysis of cellulose fibers

To examine replicas of the cellulose fibers, the samples were treated with 5% KOH overnight at room temperature, followed by 2 h of bleaching in 0.34% $NaClO_2$, buffered at pH 4.9 in 50 m*M* acetate buffer, at 80°C. These treatments were repeated three times, at which point the tissue became completely white. The purified tunic samples were transferred to an acetyl cellulose film, air dried, then unidirectionally shadowed at 45° with platinum-carbon and coated with carbon at 2×10^{-4} Pa by a BAF 400D freeze-etch apparatus (Balzers, Liechtenstein). Replicas were cleaned in a 5% sodium dichromate-50% sulfuric acid mixture (w/v) and mounted on Formvar-coated copper grids for observation with a transmission electron microscope (JEOL, JEM-2000EXII) operating at an accelerating voltage of 100 kV.

For observation of the selected area electron diffraction, purified tunic samples were mounted on carbon-coated grids after homogenization with liquid nitrogen using mortar and pestle. The electron diffraction patterns were obtained with a JEM-2000EXII transmission electron microscope operating at an accelerating voltage of 100 kV.

Table 1

Tunic Cuticle Structures in Thaliacea

Species[a]	Cuticular protrusions[b]
Subclass Pyrosomata	
Order Pyrosomatida	
Family Pyrosomatidae	
Pyrostremma agassizi	–
Pyrosoma aherniosum	–
Pyrosoma atlanticum	–
Subclass Myosomata	
Order Doliolida (=Cyclomyaria)	
Family Doliolidae	
Dolioletta gegenbauri (gono.)	–
Doliolum nationalis (gono.)	0
Order Salpida (=Desmomyaria)	
Family Salpidae	
Cyclosalpa affinis (agg.)	N
Cyclosalpa polae (agg.)	0
Cyclosalpa quadriluminis forma *parallela* (agg.)	N
Iasis zonaria (agg.)	–
Metcalfina hexagona (sol.)	–
Pegea confoederata (agg.)	N
Salpa fusiformis (sol.)	–
Salpa fusiformis (agg.)	–
Thalia cicar (sol.)	0
Thalia democratica (sol.)	+
Thalia orientalis (sol.)	+
Thetys vagina (agg.)	+
Traustedtia multitentaculata (agg.)	0
Retteriella retracta (sol.)	N

[a] gono. = gonozooid; agg. = aggregate zooid; sol. = solitary zooid.

[b] 0 = cuticular layer was not observed clearly; N = cuticular layer was not observed (too lucent or bad preservation). A plus sign indicates the presence of minute protrusions. A minus sign indicates the absence of minute protrusion (the surface of the cuticle is flat).

Results

Tunic morphology

All of the species examined in this study have a transparent, gelatinous tunic that covers the epidermis (Fig. 1).

The hardness of the tunic varies among species. The tunic is very soft and fragile in some species, such as *Doliolum nationalis, Cyclosalpa polae, Pegea confoederata,* and *Retteriella retracta.* The soft tunic was usually only lightly stained in both light and electron microscopic preparations, suggesting that the structural components of the tunic are present in low density in these species. The thickness of the tunic also varies from one species to another: in some species it has a thickness of several millimeters or more (*e.g., Pyrosoma aherniosum, Metcalfina hexagona,* and *Thetys vagina*), whereas in others (*Dolioletta gegenbauri* and *Doliolum nationalis*) it is a thin sheath of 1–2 μm. However, the thickness we measured includes substantial error attributable to shrinkage of the specimens during fixation, embedding, and sectioning. In all of the species examined, bacteria were rarely found within the tunic.

In the salps and doliolids (species of the subclass Myosomata), we found many fewer tunic cells than in the ascidian species (Fig. 2A). The sparsely distributed tunic cells are amoeboid-shaped, with extending pseudopodia (Fig. 2, B and C). In contrast, the pyrosomas have many tunic cells of several types (Fig. 3), one of which forms a cellular network in the tunic (Fig. 4A). In histological sections, elongated forms of tunic cells that appear to correspond to cells of this network form a line (Fig. 4, B and C). The network probably occurs in a specific layer in the tunic.

Salps have two forms of zooids in their life cycle: solitary (asexual) and aggregate (sexual) zooids. We examined the tunic of both forms in *Salpa fusiformis.* Although the tunic shape differs between the two forms and the tunic is usually thicker in the solitary zooids, there are no prominent differences in the morphology of tunic cells or in the fine structure of the tunic cuticle.

Tunic cuticle

The tunic cuticle is an electron-dense layer covering the tunic matrix. In some of the species that have a very soft tunic, we could not clearly distinguish the cuticular layer or the tunic matrix, or both, in thick and thin sections (Table 1). Perhaps the stainability and electron density of the cuticular layer were too low to be detected or the tunic and cuticle were so fragile that they were poorly fixed or broken in these specimens.

The thickness of the cuticular layer is about 10–20 nm in 8 of 10 species in which we clearly observed the fine structure of the tunic cuticle (Fig. 5, A–E). The other two species, *Iasis zonaria* and *Thetys vagina,* have a cuticular layer of 0.5–1.0 μm thick, including a subcuticular layer (Fig. 5F). The minute cuticular protrusions (about 50 nm in height or less) were seen only in *Thalia democratica, Thalia orientalis,* and *Thetys vagina* (Fig. 5, E and F; Table 1). In general, when the tunic is harder, the cuticular layer is thicker and the fibrous components of the tunic matrix are stained more densely.

Cellulose fibers

Figure 6 shows replica images (A, C, E, and G) and electron diffractograms (B, D, F, and H) of purified tunic fibers in *Pyrosoma atlanticum* (A and B), *Doliolum nationalis* (C and D), *Iasis zonaria* (E and F), and *Pegea confoederata* (G and H). The electron diffractograms with three to five spots (110, $1\bar{1}0$, 200, 002, and 004) in each figure indicate that the tunic of pyrosomas, doliolids, and salps contains cellulose I microfibrils with high crystallinity. No super-lattice reflections originating from triclinic crystalline cellulose (Iα) were observed. In some specimens, a 002 reflection spot originating from monoclinic crystalline cellulose (Iβ) was clearly observed (Fig. 6D). The diffractograms obtained from *Pyrostremma agassizi, Dolioletta gegenbauri, Salpa fusiformis,* and *Retteriella retracta* are essentially identical to those shown in Figure 6. The 110 diffraction spot of *Pyrosoma atlanticum* was often stronger than $1\bar{1}0$ (Fig. 6B). *Pyrosoma atlanticum* and *Doliolum nationalis* have cellulose microfibrils of 20-nm mean width (Fig. 6, A and C), whereas the microfibrils of salps are 18-nm mean width (Fig. 6, E and G). Bundles with two to six cellulose microfibrils were often observed in specimens of *I. zonaria* and *P. confoederata* (Fig. 6, E and G, arrowheads).

Discussion

The functions of the tunic would be very different between benthic and pelagic forms of tunicates. In benthic environments, many organisms are concentrated at high densities and diversities, so the primary functions of the tunic of benthic tunicates would be protection against predators, including bacterial infections, and attachment to the substratum. The tunic might also assist in competition for space. Therefore, benthic tunicates would benefit from having a thick, hard tunic containing many tunic cells with a variety of functions that contribute to body protection. In contrast, pelagic tunicates do not need a tunic for settlement or for occupying space. Hard, heavy tunics like those possessed by ascidians would be unsuitable for maintaining the neutral buoyancy of pelagic tunicates, even though they might be protective. Moreover, since pelagic tunicates are heavily preyed upon by sight predators such as fish, a thin transparent tunic that transmits light would be a distinct advantage to the pelagic forms. Thus, it is reasonable for pelagic tunicates to posses relatively soft, fragile tunics.

Whereas the salps and doliolids examined in this study contained few tunic cells, pyrosomas had relatively high numbers of tunic cells, comparable to the numbers found in ascidians. If one assumes that tunic cells have evolved primarily for protection against predators and tend to be

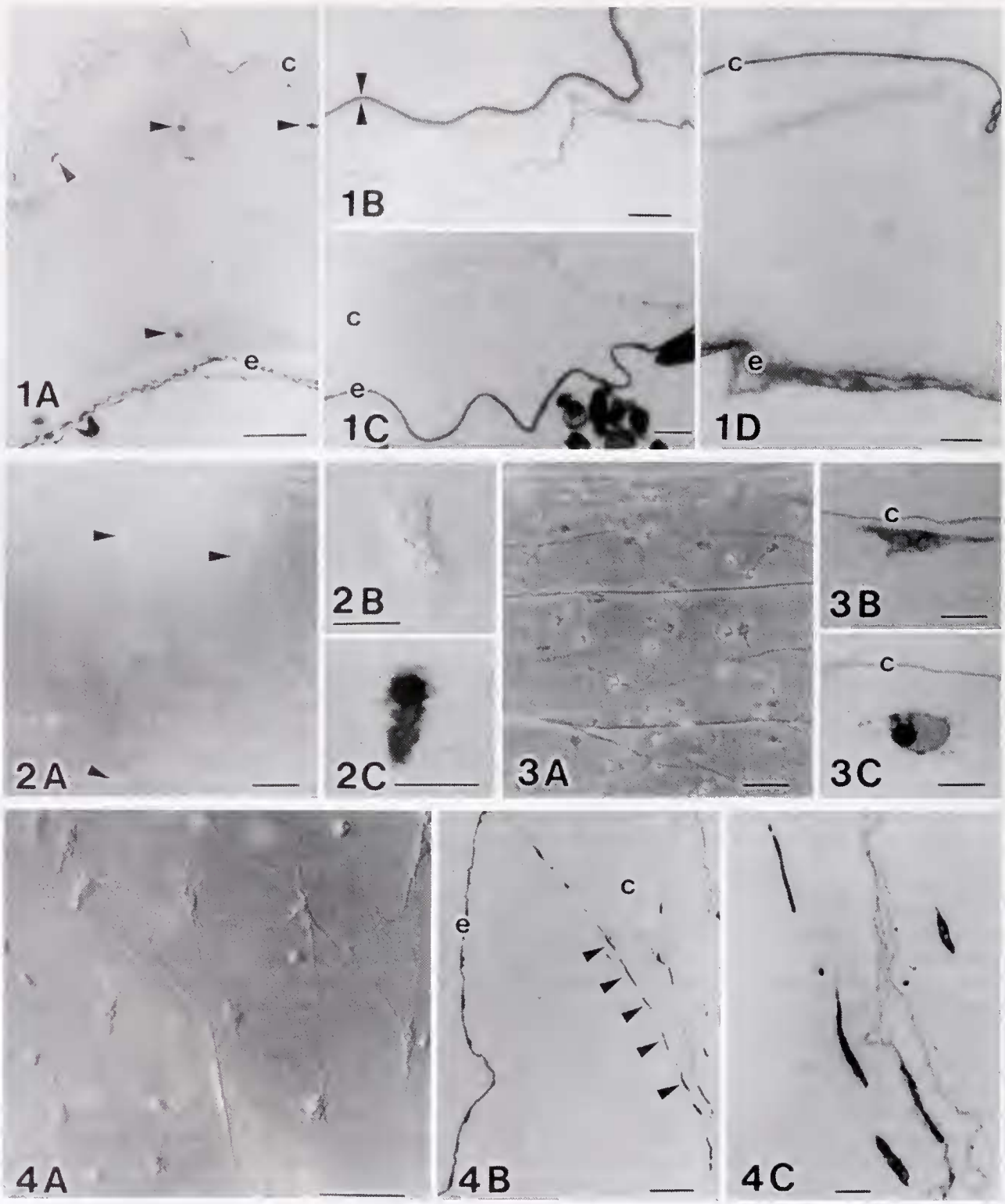
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Figure 1. Histological sections of the tunic stained with toluidine blue. The tunic matrix fills the space between the tunic cuticle (c) and the epidermis (e). (A) *Pyrosoma atlanticum.* Arrowheads indicate some tunic cells. (B) *Dolioletta gegenbauri* (gonozooid) has a very thin tunic layer (indicated by two arrowheads). (C) *Cyclosalpa polae* (aggregate zooid). (D) *Thalia orientalis* (solitary zooid). Scale bars = 50 μm (A), 10 μm (B–D).

Figure 2. Tunic cells of *Salpa fusiformis* (aggregate zooid). Tunic cells (arrowheads) are sparsely distributed in the tunic (A, phase contrast). Tunic cells are amoeboid-shaped with pseudopodia (B, Nomarski differential interference contrast; C, histological section). Scale bars = 50 μm (A), 10 μm (B and C).

Figure 3. Tunic cells of *Pyrostremma agassizi.* Several types of tunic cells are distributed in the tunic (A, Nomarski differential interference contrast; B and C, histological sections). c, tunic cuticle. Scale bars = 50 μm (A), 10 μm (B and C).

Figure 4. Multipolar tunic cells form a cellular network in the tunic of *Pyrostremma agassizi.* (A, Nomarski differential interference contrast). Tunic cells (arrowheads) forming a line are occasionally found in the tunic and probably correspond to the cellular network (B, histological section; C enlargement of B). c, tunic cuticle; e, epidermis. Scale bars = 50 μm (A and B), 10 μm (C).

well developed in animals that lack the ability to escape, these cells should be more important to pyrosomas than to doliolids and salps. Although all three groups use jet propulsion, pyrosomas swim slowly, using the water streams from the feeding currents of each zooid, whereas doliolids and salps swim quickly, using water pulses produced by the

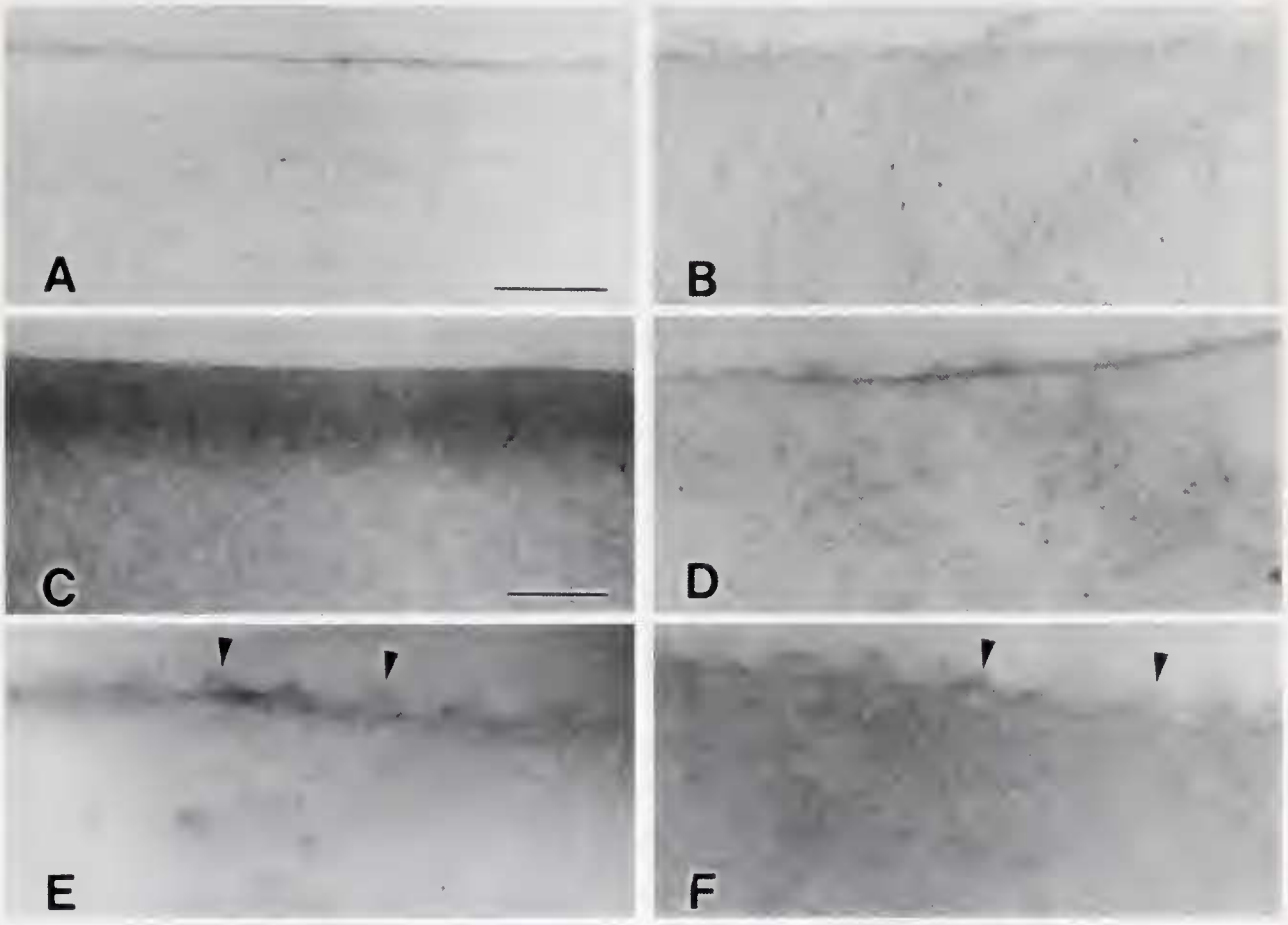

Figure 5. Transmission electron microscopy of the tunic cuticle of some thaliaceans. (A) *Pyrosoma atlanticum.* (B) *Dolioletta gegenbauri* (gonozooid). (C) *Iasis zonaria* (aggregate zooid). (D) *Metcalfina hexagona* (solitary zooid). (E) *Thalia democratica* (solitary zooid). (F) *Thetys vagina* (aggregate zooid). Arrowheads in E and F indicate cuticular protrusions. Magnifications of A, B, and D–F are identical. Scale bars = 0.2 μm (A), 1 μm (C).

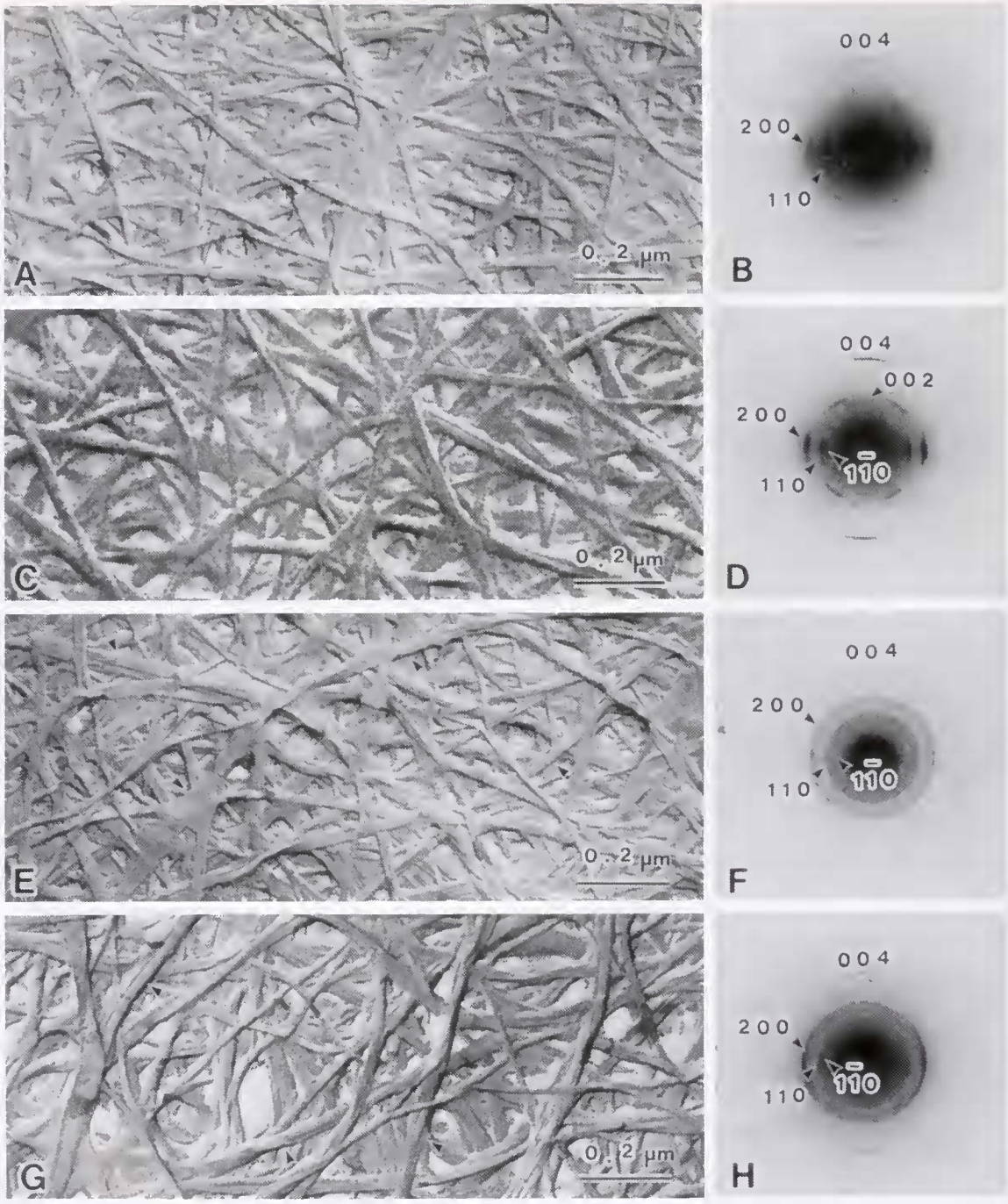

Figure 6. Replication images of purified cellulose microfibril (A, C, E, and G) and its electron diffractogram (B, D, F, and H) of *Pyrosoma atlanticum* (A and B), *Doliolum nationalis* (C and D), *Iasis zonaria* (E and F), and *Pegea confoederata* (G and H). All figures are of the same magnification. The diffractograms show that all specimens are composed of cellulose I microfibrils with high crystallinity. Bundles with two to six cellulose microfibrils were often observed in *I. zonaria* and *Pegea confoederata* (E and G, arrowheads).

rapid contraction of circular muscle bands responding to stimulation (*e.g.*, Bone and Trueman, 1983, 1984; Nishikawa and Terazaki, 1994; Bone, 1998).

The tunic cells in salps and doliolids have an amoeboid shape and many pseudopodia, characteristics that are suggestive of their motility within the tunic. Because they are very similar in morphology to the tunic phagocytes of some ascidians (*cf.* Hirose *et al.*, 1994, 1996a, b), these cells might be phagocytes. Among several types of tunic cells in pyrosomas, one type forms a cellular network. This network

might facilitate coordination among the zooids in a colony. A cellular network has been described in the tunic of some colonial ascidians; it seems to be involved in impulse conduction (Mackie and Singla, 1987) and tunic contractility in response to wounding (Hirose and Ishii, 1995; Hirose *et al.*, 1997a). Although bacteria are often found within the tunic of some ascidians (*cf.* Hirose and Saito, 1992; Hirose *et al.*, 1996a), they are rarely found within the tunic of pelagic tunicates. Perhaps the tunic of the pelagic species contains some antibiotic substances, or maybe bacterial infections are less common in the habitat of the pelagic tunicates than in that of the sessile forms. If some tunic cells of pelagic tunicates are phagocytic, they may help to keep the tunic sterile.

To date, the fine structure of the tunic cuticle has been described in 116 ascidian species covering all of the families and subfamilies of the class Ascidiacea except for the phlebobranch families Octacnemidae and Plurellidae (Hirose *et al.*, 1990, 1992, 1997b). The presence of cuticular protrusions appears to have a phylogenetic significance in Ascidiacea, because there is a general stability of the character-state distribution (presence or absence) within the families or subfamilies in the traditional classification. The authors concluded that the common ancestor of ascidians lacked cuticular protrusions and that the protrusions possibly emerged independently in several lineages (Hirose *et al.*, 1997b). With respect to ascidian phylogeny, pelagic tunicates can be considered the "out-group." Our results tend to support this concept, because many of the examined species do not have cuticular protrusions. The common ancestor of tunicates probably did not have cuticular protrusions and this character may have been independently acquired in some lineages of ascidians and thaliaceans. If cuticular protrusions emerged repetitively and became fixed in certain tunicate lineages, these protrusions should have some adaptive purpose that is as yet unknown.

Using electron diffraction, the existence of cellulose was confirmed in the tunic of three groups of pelagic tunicates: pyrosomas, doliolids, and salps. The tunic of these pelagic tunicates consisted of cellulose I microfibrils with high crystallinity and large dimensions and belonging almost entirely to the cellulose Iβ allomorph. These features are similar to those of the cellulose found in ascidians (Yamamoto *et al.*, 1989; Van Daele *et al.*, 1992; Kimura and Itoh, 1996; Okamoto *et al.*, 1996), suggesting that cellulose synthetic ability is an inherited characteristic common to ascidians and thaliaceans. The tunic cellulose in thaliaceans may be synthesized by terminal complexes (TCs) in the plasma membrane of epidermal cells, as occurs in ascidians (Kimura and Itoh, 1996). The visualization of TCs in these thaliaceans is required for further discussion of the evolution of cellulose synthesis in tunicates.

In this study, we did not examine appendicularians, the other member of the pelagic tunicates. Adult appendicularians do not have integumentary tissue outside the epidermis, but they secrete a mucous substance that forms their "house," which is a feeding apparatus. In embryonic and juvenile stages, the entire animal is covered by an acellular membrane, but it is not certain whether the membrane is equivalent to the tunic (Fenaux, 1998).

The tunic is a synapomorphic character in tunicates, and it is generally believed to have evolved monophyletically. Our present results—demonstrating that the tunic morphology and cellulosic components are fundamentally the same in ascidians, pyrosomas, doliolids, and salps—are consistent with that view. The numbers of tunic cells in doliolids and salps are much smaller than those in ascidians and pyrosomas; this difference could reflect different functions of the tunic as an integumentary tissue. Thus, perhaps the tunic has become diversified in its properties and functions, thereby making it an attractive model for studying the evolution of adaptive tissue functions.

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